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Tapasin's protein interactions in the rainbow trout peptide-loading complex

Lital Sever, Nguyen T.K. Vo, Niels C. Bols, Brian Dixon*

Department of Biology, University of Waterloo, 200 University Ave W. Waterloo, Ontario, N2L 3G1, Canada

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ABSTRACT

Major histocompatibility complex (MHC) class I receptors play a key role in the immune system by presenting non-self peptides to T cell lymphocytes. In humans, the assembly of the MHC class I with a peptide is mediated by machinery in the endoplasmic reticulum referred as the peptide loading complex (PLC). Although, the identity of the PLC has been widely explored in humans, this complex has not been characterized in fish. Co-immunoprecipitation and mass spectrometry analysis revealed that the protein-protein interactions which exist in the human PLC are conserved in the monocyte/macrophage rainbow trout cell line (RTS11), in particular the interaction of tapasin with the transporter associated with antigen processing (TAP), MHC class I and ERp57. Importantly, a 20 kDa tapasin version that contains an intact C and N terminal domains was found to associate with ERp57 and form a 75 kDa heterodimer. These results suggest a possible novel alternative spliced version of tapasin may regulate the formation of the peptide-loading complex in teleosts.

1. Introduction

The recognition of intracellular pathogens such as viruses by cytotoxic T cell lymphocytes ($CD8^+$) is mediated through MHC class I receptors on the cell surface of all nucleated cells where they present either self or foreign peptides (Blum et al., 2013). The MHC class I receptor is composed of three essential components: the polymorphic heavy chain, β2 microglobulin and a peptide of either self or foreign origin. Lack of any of these three, results in poor stability and degradation of the MHC class I receptor (Pamer and Cresswell, 1998; Wearsch and Cresswell, 2008). Peptides to be loaded into the MHC class I receptor are mainly generated by proteasomal cleavage of native proteins. These peptides are then delivered from the cytosol into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP; Hulpke and Tampe, 2013). Once the peptides reach the ER they are destined to bind to the MHC class I, which needs to achieve optimal folding in the presence of a changing repertoire of peptides (Peaper and Cresswell, 2008).

In humans, the lectin chaperone calnexin associates with the MH class I heavy chain during its early folding and recruits the thiol-disulfide oxidoreductase, ERp57. Subsequently, upon assembly with β2 microglobulin, the MHC class I heavy chain is incorporated into the peptide-loading complex (PLC) containing TAP, the lectin chaperone calreticulin and a disulphide linked conjugate of tapasin and ERp57 (Lindquist et al., 2001; Peaper et al., 2005; Rizvi and Raghavan, 2010). Tapasin is a keystone component in the PLC that forms a structural bridge between TAP and the MHC class I (Sadasivan et al., 1996; Momburg and Tan, 2002). This protein contains an immunoglobulin constant domain, a transmembrane domain and a cytoplasmic domain with ER retrieval sequence (Ortmann et al., 1997). One of its first proposed functions was to stabilize TAP and increase peptide flow to the ER (Lehner et al., 1998; Raghuraman et al., 2002), however subsequent studies showed that a soluble tapasin version which does not interact with TAP can still facilitate the recruitment of the MHC class I and peptide binding (Lehner et al., 1998). Other studies focused on the role of tapasin as a "peptide editor" (Howarth et al., 2004; Chapman and Williams, 2010), which optimizes the selection of peptides that are loaded on the MHC class I. Through a cell free system approach, it was demonstrated that recombinant tapasin-ERp57 conjugates, but not recombinant tapasin alone, can effectively recruit the MHC class I and support binding and the selection of high affinity peptides (Wearsch and Cresswell, 2007).

The first teleost major histocompatibility gene fragments were isolated from carp (Hashimoto et al., 1990) and genomic linkage

* Corresponding author.
E-mail address: bdixon@uwaterloo.ca (B. Dixon).

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studies revealed a surprising genetic architecture in which the class I and II major histocompatibility genes do not form the complex seen in other vertebrates, and therefore are referred as MH (major histocompatibility) genes (Stet et al., 2003; Phillips et al., 2003). The teleost genes involved in antigen processing, delivery and selection such as TAP, the proteasome induced subunits LMP2 and LMP7 and tapasin have been characterized and are usually linked to the MH class I genes in teleost fishes (Takami et al., 1997; Hansen et al., 1999; Landis et al., 2006). Promoter analysis of MH class I and tapasin genes revealed the presence of a putative interferon regulatory factor binding site and an interferon-γ binding site that supported their transcriptional induction by interferons during salmonid viral infection (Hansen et al., 1999; Jørgensen et al., 2006). In addition, the ER chaperones: calreticulin, ERp57 and calnexin, which are involved in the formation of the PLC, have been identified and studied in several teleost fishes (Kales et al., 2004; Fuller et al., 2004; Sever et al., 2013a, 2014b). To date, no evidence has been published describing a possible PLC in a non-mammalian vertebrate mainly due to the lack of antibodies. This report shows, for the first time, the unique and conserved protein interactions that may constitute the PLC in teleost fish, as well as the possible unique mechanisms that regulate the formation of this PLC.

2. Material and methods

2.1. Fish cell cultures

A semi-adherent monocyte/macrophage RTS11 cell line originally established from rainbow trout spleen (Ganassin and Bols, 1998), was maintained in Leibovitz's L-15 medium (ThermoFisher Scientific, Nepean, ON) with 20% fetal bovine serum and 150 U/mL of penicillin and 150 mg/mL streptomycin (ThermoFisher, Nepean, ON) in 75 cm^2 culture flasks. Two adherent rainbow trout cell lines: RTmt from the testis and RTovarian fluid from the ovarian fluid (Vo NTK and Bols NC, unpublished) were grown in room temperature in L-15 with 10% FBS. Long-term leukocyte cultures that were rich in dendritic cells and macrophages were grown from caudal fin organ explants from rainbow trout in L-15 with 30% FBS (Vo NTK and Bols NC, unpublished). All rainbow trout cell cultures were maintained at 20 °C. The fathead minnow-derived adherent epithelial EPC cell line (Winton et al., 2010) was maintained in L-15 medium with 10% FBS in 75 cm^2 culture flasks at 26 °C. All adherent cell lines were sub-cultured on a weekly basis by trypsin (Lonza, Allendale, NJ).

2.2. VHSV IVa propagation and infection of RTS11 cells for immunoprecipitation

A North American group viral hemorrhagic septicemia virus IVa isolate (VHSV IVa) obtained from Pacific herring was routinely propagated on EPC cultures in L-15 medium with 2% FBS at 14 °C as described (Sever et al., 2014a). Briefly, once the confluent cell monolayer was completely destroyed, the virus-containing conditioned media was spun at 4500 × g for 5 min at 4 °C. The supernatants were filtered through 0.2-μm membranes (Pall Corporation, Mississauga, ON) and stored in aliquots at −80 °C for later use. Viral titers were determined by $TCID_{50}$/mL assays as previously described (Pham et al., 2013). For cell infection, approximately, 1×10^8 RTS11 cells were challenged with 1×10^8 $TCID_{50}$/mL of VHSV IVa in L-15 media supplemented with 2% FBS. In the control (non-infected) cultures, the virus was absent. Both cultures were incubated for 7 days at 14 °C. Cells were then collected and centrifuged at 500 × g for 4 min at 4 °C. Subsequently, cell pellets were incubated in 5 mL of ice-cold PBS containing 10 mM of methyl methanethiosulfonate (MMTS: ThermoFisher, Nepean, ON) for 3 min followed by quick centrifugation at 500 × g.

2.3. Immunoprecipitation

RTS11 and primary fin cultures (1×10^8 cells) were collected and incubated for 2 min with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) containing 10 mM of MMTS (ThermoFisher Scientific, Nepean, ON). Subsequently, cells were centrifuged at 500 × g for 3 min and lysed with 1% digitonin (Sigma Aldrich, St. Louis, MO) containing 10 mM MMTS, 50 mM of Tris-HCl, 150 mM of NaCl supplemented with 1× protease inhibitors (Roche, Mannheim, Germany). Cells were rotated at 4 °C for 30 min followed by centrifugation at 10,000 rpm for 30 min to remove cellular debris. Immunoprecipitation was performed using either 1:50 affinity purified tapasin antibody (Sever et al., 2013a); 1:50 pre-immune tapasin rabbit serum; 1:200, anti-MH polyclonal antibody (Kawano et al., 2010) or 1:200 of anti TAP1 antibody (SAB2102370: Sigma Aldrich, St. Louis, MO). Antibodies were rotated with lysates overnight at 4 °C, followed by the addition of 50 μL of protein A agarose beads (Sigma Aldrich, St. Louis, MO) for 1 h. The beads were spun down by centrifugation at 3000 rpm and subsequently washed three times with 500 μL of 0.1% of digitonin lysis buffer. Primary elution for tapasin pull downs was performed by adding 100 μM tapasin peptide in 0.1% of digitonin lysis buffer, which corresponds to the last 21 aa of trout tapasin's C terminal domain. Trout tapasin's protein sequence was obtained from the GeneBank accession number (NP_001118025.1).

Primary elution was followed by a second boiling elution in 50 μL of 2× SDS sample buffer. Boiled elutions were separated on a 10% SDS page gel, transferred to a nitrocellulose membrane and probed with either anti MH class I (1:400) antibody or (1:200) anti-tapasin antibody for 1 h. Detection was performed using anti-rabbit IgG VeriBlot (HRP) diluted with 1:800 of skim milk and clarity™ (Roche, Mannheim, Germany) according to manufacturer's instructions.

Tapasin peptide elutions were separated on a 10% SDS page gel and stained with Coomassie blue (0.1% Coomassie blue, 40% methanol, 10% acetic acid, 50% Milli-Q water) for 1 h followed by addition of destaining solution (5% methanol, 7% acetic acid, 88% Milli-Q water) overnight at 4 °C. Proteins with relative molecular weight of 75 kDa, 55 kDa and 25 kDa were excised and stored at 4 °C in 1% acetic acid in Milli-Q water, until ready for digestion.

Immunoprecipitations for tapasin and MH class I were repeated 4 times (see Supplementary Figs. 1 and 2 for examples). IPs for tapasin and ERP57 were repeated 3 times. The IP for the tapasin and TAP interaction was only performed once so this result might be preliminary, but TAP peptides were detected in small quantities in the mass spectrometry of tapasin bands, corroborating this interaction.

2.4. Western blotting

For the detection of the interaction between ERp57 and tapasin, non-stimulated 2×10^6 RTS11 cells were collected and incubated for 2 min with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) containing 10 mM of MMTS (ThermoFisher Scientific, Nepean, ON). Subsequently, cells were lysed for 30 min with 1% digitonin (Sigma Aldrich, St. Louis, MO) containing 10 mM of MMTS, 50 mM of Tris-HCl, 150 mM of NaCl supplemented with 1× protease inhibitors (Roche, Mannheim, Germany). Supernatants were collected after centrifugation at 13,000 rpm for 20 min. Protein samples were prepared under reducing conditions with β-mercaptoethanol or non-reducing conditions, without β-mercaptoethanol, and loaded onto a 10% SDS-page gel and probed with 1:1000 anti-ERp57 antibody (Sever et al., 2013b) or 1:200 anti-tapasin antibody (Sever et al., 2013a). Detection was performed using anti-rabbit IgG VeriBlot (HRP) diluted with 1:800 of skim